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METHOD FOR THE DETERMINATION OF TRIGLYCERIDES IN PROTEIN FRACTIONS, ENZYME SOLUTION FOR CARRYING OUT THE METHOD AND USE OF THE METHOD

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METHOD FOR THE DETERMINATION OF TRIGLYCERIDES IN PROTEIN FRACTIONS, ENZYME SOLUTION FOR CARRYING OUT THE METHOD AND USE OF THE METHOD

[Verfahren zur Bestimmung von Triglyceriden in Proteinfraktionen, Enzymlösung zur

Durchführung des Verfahrens sowie Verwendung des Verfahrens]

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Petition for examination has been submitted in accordance with § 44 of the patent law.

The following information is taken from documents submitted by the applicant.

Description /

The present invention relates to a method for the determination of triglycerides in protein fractions.

Coronary heart disease continues to be the main cause of death in western industrialized nations. While the significance of cholesterol as a risk factor for coronary heart disease is

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* [Numbers in the right margin indicate pagination of the original text.]

generally known, in that connection, one also takes into account an evaluation of protein associated triglycerides, which belong to the so-called lipoproteins in the blood serum.

The serum protein fractions are subdivided based on differences in density into lipoproteins with very low density ("very low density lipoproteins," hereafter abbreviated VLDL), lipoproteins with low density ("low density lipoproteins," LDL) and lipoproteins with high density ("high density lipoproteins," HDL).

For diagnosing vascular diseases, such as coronary heart disease, peripheral arterial occlusion disease and microangiopathological changes, the triglyceride content in the individual protein fractions as well as the relative proportions of the protein fractions to each other are important. In particular, for the LDL fraction it is assumed that a high triglyceride content is associated with coronary heart disease.

Simple method and means for the determination of the triglyceride content in individual protein fractions, in particular in the LDL fraction, are not available to date.

The precipitation technique is designed primarily for the determination of the triglyceride content in high density serum proteins (HDL). The selective precipitation of LDL triglycerides has been attempted, however the cure precipitation technique presents a drawback, namely the fact that considerable quantities of VLDL coprecipitate with the LDL protein fraction, so that a differentiation of the triglyceride content into the appropriate serum protein groups is only possible with difficulty (see R. Siekmeier et al. in Clin. Chim. Acta 177, pp. 231-230: "Precipitation of low density lipoproteins with sulfonated polyanions" (1988), R. Siekmeier et al. in Clin. Chem. 36, pp. 2109-2113: "Insufficient accuracy and specificity of polyanion precipitation methods for the quantitation of low density lipoproteins" (1990), and M. Nauck et

al. in Klin. Lab. 40, pp. 167-176: Measurement of LDL and VLDL cholesterol with precipitation techniques. A comparison with the ultracentrifugation method" (1994).

Therefore, in practice LDL triglycerides have been determined by a sequential ultracentrifugation according to their density in the ultracentrifuge, which has to be run for 48 h to obtain the LDL fraction, or the LDL triglycerides are determined by a shortened combined method using ultracentrifugation and precipitation. In the last mentioned, relatively selective separation, the VLDL fraction is first separated with the ultracentrifuge (duration approximately 24 h), and the LDL fraction which then remains is precipitated more or less selectively using appropriate agents (Manual of Laboratory Operation (1979); DHEW No (NIH) 75-628 National Heart and Lung Institute; Lipid Research Clinics Program, Bethesda, MD, USA, pp. 1-74). Then, the quantity of LDL triglyceride is calculated from the triglyceride concentration before and after the LDL precipitation.

For this combined ultracentifugation/precipitation method, the drawback is that, besides the fact that it is very time consuming, it also requires a high level of expenditure for equipment, which, as a rule, is prohibitive for a routine laboratory. However, if one considers the LDL triglyceride concentration to be particularly valid in terms of preventing coronary heart disease, then it is precisely routine determination of LDL triglycerides which is desirable.

The invention is therefore based on the problem of improving the determination of triglycerides in protein fractions in such a manner that it can also be used in the routine diagnosis in cardiac infarct prevention.

This problem is solved by a method for the determination of triglycerides in protein fractions, which is characterized by the following steps:

a) electrophoretic separation of the protein fractions in its own matrix,

b) enzymatic cleavage of the triglycerides, and

c) determination of the glycerol produced by step b).

An additional object of the present invention consists in preparing an enzyme solution according to Claim 15, which is particularly well suited as a means to carry out the above-mentioned method.

An additional object of the present invention is the use of the above-mentioned method for in vitro diagnosis of vascular diseases. This includes, in particular, the diagnosis of coronary heart disease, another macro- and micro-angiopathological vascular diseases. Diagnosis also includes the evaluation of the risk of developing these diseases.

Advantageous embodiments of the present invention can be obtained from the secondary claims.

The special advantages of the method according to the invention consists of the simple and, if desired, quantifiable determination of triglycerides in protein fractions with low expenditure for equipment. This makes the method according to the invention particularly advantageous for the determination of the triglycerides of the LDL fraction in a routine laboratory.

Triglycerides are esters of the triple //??// alcohol glycerol with fatty acids. The quantity of triglycerides is determined from the quantity of esterified glycerol in the triglycerides, where the triglycerides in combination with the proteins are separated electrophoretically in an appropriate matrix and then cleaved enzymatically.

Using the method according to the invention it is not only possible to determine the triglyceride content in the LDL fraction, one can also, without additional expenditure, determine the triglyceride content in other protein fractions, for example, the VLDL fraction and the HDL

fraction. It is also highly advantageous that free, that is not protein bound, triglyceride or glycerol, in contrast to the other protein associated triglycerides, does not migrate due to /3 electrophoresis with the protein fractions, and thus it does not interfere with the triglyceride determination of the protein fractions. For that reason, it is also possible to determine, additionally, the concentration of free, unesterified glycerol or free triglyceride.

For the enzymatic cleavage of the triglycerides, appropriate esterases are suitable, for example, the enzyme lipase (triacyl glycerol acyl hydrolase, EC 3.1.1.3). In view of the fact that the lipoprotein fractions contain, besides triglycerides, other fatty substances, in particular cholesterol esters, one can achieve a special effect by using cholesterol esterase (EC 3.1.1.13) for cleaving the triglycerides. This leads to the simultaneous cleavage of triglycerides and cholesterol esters. The result is that the lipoprotein molecule is effectively denatured, and the access of the enzyme to the triglyceride substrate as well as the access of the determination reagents are simplified in the subsequent glycerol determination step of the method according to the invention.

In addition, the use of cholesterol esterase presents the advantage that, due to the simultaneous cleavage of cholesterol ester and triglycerides, both substances in the protein fractions can be determined. To determine cholesterol in protein fractions one can, for example, use the method described in DE-A-36 40 349. The determinations, which may be carried out simultaneously or successively, of both triglycerides and cholesterol in a sample increases the validity of the diagnosis of vascular diseases, in particular coronary heart disease, as well as the associated risk evaluation.

The glycerol produced by enzymatic cleavage can be determined by incubating the separation matrix, for example, a gel matrix, in an enzyme solution, which contains, as enzymes,

glycerol kinase and //3-//glycerol-3-phosphate dehydrogenase, resulting in the formation of a reduced acceptor of reduction/oxidation equivalents, which is determined by a detection reaction. The enzyme solution used also contains the appropriate substrates and cofactors. Thus, glycerol is converted with a donor of energy rich phosphate groups, such as, for example, adenosine triphosphate (ATP) and the enzyme glycerol kinase (EC 2.7.1.30), into glycerol-3-phosphate, and the glycerol-3-phosphate is converted with the acceptor of reduction/oxidation equivalents, for example, nicotin adenine dinucleotide (NAD) and the enzyme glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), into dihydroxyacetone phosphate, where the reduced acceptor of reduction/oxidation equivalents is formed (in the case of NAD, this is NADH), which can be determined by appropriate detection reactions, which in themselves are known.

In a particularly preferred embodiment of the present invention, the enzymes solution with which the matrix is incubated, additionally contains the enzymes triosephosphate isomerase and gyceraldehyde-3-phosphate dehydrogenase, resulting in the additional formation of a reduced acceptor of reduction/oxidation equivalents, which is determined by the detection reaction. This means, in addition to the above-mentioned intermediates, the forming dihydroxyacetone phosphate is converted with the enzyme triosephosphate isomerase (EC 5.3.1.1) into glyceraldehyde-3-phosphate, and the glyceraldehyde-3-phosphate is converted with the acceptor of reduction/oxidation equivalents (for example, NAD) and the enzyme glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) into glycerate-3-phosphate, resulting in the formation of an additional reduced acceptor of reduction/oxidation equivalents (in this example NADH), which in turn can be determined by the above-mentioned, appropriate detection reaction. In this manner, one obtains from one molecule of triglyceride or glycerol, two molecules of the reduced acceptor of reduction/oxidation equivalents (for example, NADH).

Thus, in principle, this measure allows a doubling of the sensitivity of the method according to the invention for the determination of triglycerides in protein fractions.

The above described steps or reactions of the method according to the invention occur on or in the matrix which is used for the electrophoretic separation of the protein fractions, where the enzymatic cleavage of the triglycerides and the determination of the glycerol produced in the process advantageously occur simultaneously.

As matrix base material, one can consider using, first of all, a gel, where an agar gel or a crosslinked polyacrylamide gel is particularly appropriate. Advantageously, an agar gel with an agar concentration of 0.5-2.0 wt%, in particularly 1.0-1.5 wt%, is used, because it allows a good separation of the lipoprotein fractions. The addition of albumin to the gel, in addition, has an advantageous effect on the separation capacity. Combined gels made of polyacrylamide and agar are also suitable for this method.

The detection reaction for the determination of the formed reduced acceptors of reduction/oxidation equivalents such as NADH, which allows conclusions concerning the presence of the released glycerol and thus concerning the original triglycerides in a given protein fraction, is usually carried out by densitometry. By this measure, the triglyceride content can easily be quantified in the individual protein fractions separated by electrophoresis.

The detection method by densitometry is particularly well suited, in which the reduced acceptor of reduction/oxidation equivalents is detected with a color indicator, by reducing a dye with an electron coupler, where the dye precipitates and thereby can be determined by densitometry. This means that the reduction equivalents of the reduced acceptor of reduction/oxidation equivalents (such as, for example, NADH), are transferred by an appropriate electron coupler to suitable dye, which is water insoluble in the reduced form and thereby

precipitates in the gel or on the gel. Suitable electron couplers are, for example, the enzyme diaphorase (EC 1.8.1.4); another example of an electron coupler to be used is phenazine methosulfate (PMS). Suitable dyes, which precipitate the above reduction and which can be quantified by densitometry in the gel, are, for example, the tetrazolium salts. The latter, as a result of reduction, are converted into the corresponding formazanes, which are water insoluble and accordingly precipitate in or on the gel. Examples of such tetrazolium salts for formazane formation are tetrazolium blue, nitroblue tetrazolium (NBT), tetrazolium violet, tetrazolium purple, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), etc. The determination by densitometry is then best carried out at the absorption maximum of the formed dye, for example, in the case of NBT or INT at a wavelength of 570 nm. /4

A particularly suitable means to carry out the method for the determination of triglycerides in protein fractions, another object of the present invention consists of making available an enzyme solution which comprises the enzymes required to carry out the conversions and, optionally, other components which are suitable for the conversions, such as substrates and cofactors. Thus the enzyme solution comprises at least the enzymes of an appropriate esterase, for example, cholesterol esterase, glycerol kinase and glycerol-3-phosphoate dehydrogenase, as well as, optionally, additionally the enzymes triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase and an electron coupler.

The enzymatic cleavage of the triglycerides and the determination of the glycerol produced according to the present invention is carried out in this preferred embodiment in a simple manner by incubating the separation matrix, which contains the electrophoretically separated protein fractions, with the mentioned enzyme solution. It is more advantageous to carry out this incubation of the matrix (for example, of a gel) in a solution, which contains,

besides the mentioned enzymes, a buffer system which is set to a pH range of approximately 7.5-9, such as glycerol buffer or tris buffer, adenosine triphosphate (ATP) as donor of energy rich phosphate groups, nicotin adenine dinycleotide [sic; dinucleotide] (NAD) as acceptor of reduction/oxidation equivalents, a calcium ion chelator such as EDTA, a magnesium salt such as $MgCl_2$, where the chelation of Ca^{2+} ions as well as the availability of Mg^{2+} ions serve to achieve an effective phosphorylation, as well as, optionally, the color indicator.

A suitable composition of the enzyme solution, which is used for the incubation of the separation matrix, contains the following components at the indicated concentrations: 0.5-60 kU/L cholesterol esterase, 0.5-50 kU/L glycerokinase, 1-500 kU/L glycerol-3-phosphate dehydrogenase, 10-3000 kU/L triosephosphate isomerase, 1-250 kU/L glyceraldehyde-3-phosphate dehydrogenase, 0.5-50 kU/L diaphorase, 50mM-1M glycylglycine, 0.5-50mM ADP, 0.5-50mM NAD, 0.1-5mM EDTA, 2-100mM $MgCl_2$, and 0.5-20mM NBT.

In addition, the enzyme solution or incubation solution can contain additional additives, for example, enzyme stabilizers, enzyme activators, such as gallic acids (cholates) and detergents (for example, genapol) for activating the cholesterol esterase, preservatives, etc.

The incubation of the gel for the enzymatic cleavage of the triglycerides and the determination of the glycerol, with respect to the incubation time and the temperature of the incubation solution, is carried out under conditions which are appropriate to achieve a densitometric determination of sufficient quantity using precipitated dye. Suitable incubation times are at least 10 min, preferably 25-35 min, and in particular 30 min. The incubation temperature is advantageously in the range of 20-40°C, preferably in the range of 25-35°C, and more advantageously approximately 30°C.

Above, a preferred embodiment was described, in which the determination of the glycerol produced after the enzymatic cleavage of the glycerides is carried out by forming, by enzymatic reactions, a reduced acceptor of reduction/oxidation equivalents. However, it is also possible to use systems, in which oxidized acceptors of reduction/oxidation equivalents are formed, where then a corresponding different dye is used, which is then precipitated by oxidation and can be quantified by densitometry in the matrix (for example, in the gel).

Alternatively, the determination can be carried out in the above described system, in which a reduced acceptor of reduction/oxidation equivalents is detected, for example, by measurement of luminosity where the flavin//e mononucleotide (FMN) is reduced with flavin mononucleotide reductase (EC 1.6.8.1), and the reduced flavin mononucleotide ($FMNH_2$) is determined by measuring the luminosity, by causing it to be converted, together with appropriate cosubstrates, such as an alkanal (for example, tetradecanal) in the presence of oxygen by the enzyme luciferase, during which process light is emitted. The determination by measuring the luminosity, which is used in this embodiment, can be carried out, for example, by running the required reactions in the separation matrix and, at the same time, by applying a light sensitive film, with screening against external light radiation, on the matrix, so that the light radiation generated by the luciferase reaction causes a blackening in the film at the corresponding places in the matrix .

As additional alternatives for the above embodiments, in which the conversions can occur for //sic; by// enzymatic cleavage of the triglyceride and for the determination of the glycerol formed in the separation matrix, the triglyceride associated protein fractions, after the electrophoretic separation, can also be transferred by appropriate means, for example, electrophoretically, or by so-called "blotting," onto a protein binding layered material, for

example, nitrocellulose paper, and then the reactions for enzymatic cleavage of the triglycerides and for the determination of the resulting glycerol, as described above, can occur consecutively.

The method according to the invention for the determination of triglycerides in protein fractions or the enzyme solution for carrying out the method is particularly well suited for in vitro diagnosis, prognosis and risk evaluation of vascular diseases, such as coronary heart disease. It can be assumed here that, in particular the small, triglyceride-rich LDL are associated with coronary heart disease and other vascular diseases.

The present invention is further explained below in the following examples, with reference to the figures in the appendix.

Figure 1 shows the conversions in a preferred embodiment of the method according to the invention,

Figure 2 shows the dose-signal representation, which was obtained by graphically plotting the protein concentration used in the method according to the invention according to a preferred embodiment against the extent of color indication (colored gel surfaces), and

Figure 3A shows a photographic representation of a gel after the determination of the protein associated triglycerides according to the method of the invention together with a /5 cholesterol determination, and Figure 3B shows the corresponding densitometric distribution.

Examples 1 and 2

Serum lipoproteins, in various quantities, were subjected as follows to an agar gel electrophoresis. The protein samples were applied to an agar gel and then subjected at 400 V and 20°C to an electrophoresis.

Then the triglyceride determination was carried out for 2 types.

In Example 1, all the reactions represented in Figure 1 by the steps (a)-(f) were carried out. The enzyme abbreviations for Figure 1 are: CE = cholesterol esterase, GK = glycerokinase, GDH = glycerol-3-phosphate dehydrogenase, TIM = triosephosphate isomerase, GADPH = glyceraldehyde 3-phosphate dehydrogenase and DP = diaphorase; P stands for phosphate, and NBT stands for nitroblue tetrazolium.

Thereafter, the agar gel was incubated in an enzyme solution with the protein fractions, whose solutions are listed below, to run the reactions represented in Figure 1. The enzyme substrate solution was heated to incubation temperature shortly beforehand, and the gel was incubated for 30 min at 30°C.

Composition of the enzyme substrate solution:

Glycylglycine	0.2M
ATP	5mM
NAD^+	5mM
EDTA	0.5mM
$MgCl_2$	10mM
4-NBT	3mM
Cholesterol esterase (EC 3.1.1.13)	6 kU/L
Glycerokinase (EC 2.7.1.30)	4.8 kU/L
Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)	48 kU/L
Triosephosphate isomerase (EC 5.3.1.1)	300 kU/L
Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	24 kU/L
Diaphorase (EC 1.8.1.4)	4.8 kU/L

The above-mentioned formulation was produced by first adding the following quantities to stock solutions, which will be described below, to produce the substrate/dye solution:

Reaction buffer	1.2 mL
ATP	250 µL
NAD^+	250 µL
EDTA	75 µL
$MgCl_2$	30 µL
4-NBT	45 µL

In addition, the following enzymes were separately prepipetted, left on ice until shortly before use, and then added together with the above-mentioned buffer/substrate/dye mixture:

Cholesterol esterase	75 µL
Glycerokinase	30 µL
Glycerol-3-phosphate dehydrogenase	75 µL
Triosephosphate isomerase	75 µL
Glyceraldehyde-3-phosphate dehydrogenase	75 µL
Diaphorase	65 µL

The compositions of the individual stock solutions are as follows:

Reaction buffer: 6.6 g glycylglycine are dissolved in 250 mL twice distilled water and the pH is adjusted to 8.5 (glycylglycin buffer 0.2M). 81 mg $MgCl_2 \times 6H_2O$, 86 mg Na cholate and 0.5 mL Genapol® are brought up to a volume of 100 mL with glycylglycine buffer.

ATP: 60.5 mg adenosine-5'-triphosphate·$Na_2H_2 \times 3H_2O$ are dissolved in 2 mL reaction buffer (stock solution 50mM).

NAD: 27 mg β-nicotinamide adenine dinucleotide are dissolved in 1 mL reaction buffer (stock solution 40mM).

EDTA: 724 mg EDTA-Na_2 x $2H_2O$ are dissolved in 10 mL reaction buffer (stock solution 20mM).

$MgCl_2$: 2.03 $MgCl_2$ x $6H_2O$ g are dissolved in 10 mL reaction buffer (stock solution 1M).

4-Nitroblue tetrazolium (4-NBT): 50 mg 4-nitroblue tetrazolium chloride are dissolved in 250 µL 70% dimethylformamide (stock solution 20% (weight/volume)).

Enzymes: 100 U lyophilized cholesterol esterase are dissolved in 500 µL reaction buffer. 200 U lyophilized diaphorase are dissolved in 500 µL reaction buffer. All the other enzymes are used at the following enzyme activities: glycerokinase 425 kU/L, glycerol 3-phosphate dehydrogenase 1700 kU/L, triosephosphate isomerase 10,000 kU/L and glyceraldehyde 3-phosphate dehydrogenase 800 kU/L.

After the agar gel was incubated in an enzyme substrate solution having the above-mentioned composition, the incubation solution was removed by washings and the dyed bands were fixed with 10% (volume/volume) acetic acid for 30 min. The gels were then immersed in distilled water for an additional 30 min and dried. Then the red/violet colored light at a wavelength of 570 nm was evaluated by densitometry. After the fixation and the drying, the gel was still capable of being stored for months without any loss of information content. This means, the color indication is stable. Thus the evaluation for obtaining information by densitometry can also be carried out, if desired, at any later time.

In Example 2, only the steps (a)-(c) and (f) represented in Figure 1 were carried out; the steps (d) and (e) were thus omitted. For this purpose, the used quantities of the enzymes triose

phosphate isomerase and glyceraldehyde dehydrogenase were replaced by corresponding volumes of reaction buffer. Otherwise, the procedure was the same as in Example 1.

Figure 2 is a representation of the results of the determination methods used in Examples 1 and 2. On the abscissa, the quantities of the serum lipoproteins used according to the two examples can be seen, while on the ordinate, the measurements of the coloration by determination of the colored surface area (arbitrary units) are shown.

As can be seen in Figure 2, the determination method which is carried out in the presence of enzymes triose phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, presents all the steps (a)-(f) nearly twice as high sensitivity, compared to the method which is carried out without the mentioned enzymes, and thus comprises only the steps (a)-(c) and (f).

In addition, in Examples 1 and 2, an additional linear analytical area was observed, namely the graphic relation between the used triglyceride-containing protein concentrations and the resulting signal values (the color indication) was linear in a broad range of protein concentration. Thus the method in accordance to the invention is excellently suited for the quantitative determination of protein-associated triglycerides.

Example 3

In each case 1 μL of different blood serum samples is applied to an agar gel, and then subjected at 400 V and 20°C to electrophoresis. As a result, the different proteins present in the blood serum are separated into protein fractions.

Then an enzymatic cleavage of the triglycerides as well as an enzymatic determination of the resulting glycerol was carried out as in Example 1. At the same time, the cholesterol content was determined in the given samples according to DE-A-36 40 349.

Figure 3A shows the triglyceride-associated protein fractions of blood serum samples in the gel (the gel lanes bearing the indication TG (triglyceride), which have been stained according to the invention. In addition, one can see the cholesterol determination (see the lane marked with CH (cholesterol)). Figure 3B shows the corresponding densitometric distributions of the individual samples, as recorded with a densitometer.

Three lipoprotein fractions became visible, namely the LDL/TG fraction, the VLDL/TG fraction, and the HDL/TG fraction. In addition, one can recognize free glycerol as a fourth lane. Using the densitometric determination, it is possible to determine the percentage distribution of the triglycerides in the lipoprotein fractions (see right column in Figure 3B).

For an absolute determination of the triglyceride content in the individual protein fractions in the blood serum, the relative proportions of the fractions are multiplied by the total triglyceride content in the blood serum, where the total triglyceride content in the blood serum can be determined by known conventional methods.

Claims

1. Method for the determination of triglycerides in protein fractions, characterized by the following steps:

a) electrophoretic separation of the protein fractions in its own matrix,

b) enzymatic cleavage of the triglycerides, and

c) determination of the glycerol produced by step b).

2. Method according to Claim 1, characterized in that the triglycerides are cleaved with cholesterol esterase.

3. Method according to Claim 1 or 2, characterized in that the glycerol produced is determined by incubating the gel in an enzyme solution, which contains, as enzymes, glycerol-kinase and glycerol-3-phosphate dehydrogenase, resulting in the formation of a reduced acceptor of reduction/oxidation equivalents, which is determined by a detection reaction.

4. Method according to Claim 3, characterized in that the enzyme solution in addition contains the enzymes triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase, resulting in the formation of an additional reduced acceptor of reduction/oxidation equivalents, which is determined by the detection reaction.

5. Method according to Claim 3 or 4, characterized in that the reduced acceptor of reduction/oxidation equivalents is detected with a color indicator, by reduction of a dye with an electron coupler, followed by the precipitation and detection by densitometry of the dye.

6. Method according to Claim 5, characterized in that diaphorase is used as electron coupler.

7. Method according to Claim 5 or 6, characterized in that a tetrazolium salt is reduced, with precipitation of a formazan dye which is detected by densitometry at the wavelength of the dye absorption maximum.

8. Method according to one of Claims 3-7, characterized in that the enzymatic cleavage of the triglycerides and the determination of the glycerol by incubation of the gel are carried out in a solution, which contains, besides the mentioned enzymes, a buffer system to be set to a pH range of 5.7-9, adenosine triphosphate as donor of energy rich phosphate groups, nicotin adenine dinucleotide as acceptor of reduction/oxidation equivalents, a Ca ion chelator and a magnesium salt and optionally the color indicator.

9. Method according to Claim 8, characterized in that the incubation solution has the following components:

50mM to 1M glycylglycine, 0.5-50mM adenosine triphosphate, 0.5-50mM nucleotin adenine dinucleotide, 0.1-5mM EDTA, 2-100mM $MgCl_2$, 0.5-20mM 4-nitrosic blue tetrazolium, 0.5-60 kU/L cholesterol esterase, 0.5-50 kU/L glycerol kinase, 1-500 kU/L glycerol-3-phosphate dehydrogenase, 10-3000 kU/L triosephosphate isomerase, 1-250 kU/L glyceraldehyde-3-phosphate dehydrogenase and 0.5-50 kU/L diaphorase.

10. Method according to one of the preceding claims, characterized in that the enzymatic cleavage of the triglycerides and the determination of the glycerol is carried out by a treatment of the matrix for an incubation time of at least 10 min at an incubation temperature of 20-40°C.

11. Method according to one of the preceding claims, characterized in that all the steps are carried out in the matrix, where the step of enzymatic cleavage and the detection step are carried out simultaneously.

12. Method according to one of the preceding claims, characterized in that the gel is used as matrix.

13. Method according to Claim 12, characterized in that an agar rose gel, a polyacrylamide gel or a combined agar polyacrylamide gel is used.

14. Method according to one of the preceding claims, characterized in that cholesterol is additionally determined in the protein fractions.

15. Enzyme solution for carrying out methods according to any one of the preceding claims, comprising the enzymes esterase, glycerol kinase and glycerol-3-phosphate dehydrogenase.

16. Enzyme solution according to Claim 15, characterized in that it additionally contains the enzymes triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and an electron coupler.

17. Enzyme solution according to Claim 15, characterized in that it contains, besides the mentioned enzymes, a buffer system to be set to a pH range of 5.7-9, adenosine triphosphate as donor of energy rich phosphate groups, nicotin adenine dinucleotide as acceptor of reduction/oxidation equivalents, a Ca ion chelator, a magnesium salt and a color indicator.

18. Enzyme solution according to Claim 17, characterized in that it contains the following components:

50mM to 1M glycylglycine, 0.5-50mM adenosine triphosphate, 0.5-50mM nucleotin adenine dinucleotide, 0.1-5mM EDTA, 2-100mM $MgCl_2$, 0.5-20mM 4-nitroblue tetrazolium, 0.5-60 kU/L cholesterol esterase, 0.5-50 kU/L glycerol kinase, 1-500 kU/L glycerol-3-phosphate dehydrogenase, 10-3000 kU/L triosephosphate isomerase, 1-250 kU/L glyceraldehyde-3-phosphate dehydrogenase and 0.5-50 kU/L diaphorase.

19. Use of the method according to one of Claims 1-14 for the in vitro diagnosis of vascular diseases.

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//scan in Figure 1//

Key: 1 Triglycerides …

2 Glycerol + 3 free fatty acids

3 Glycerol …

4 Glycerol //-3-P// //three times//

5 Dihydroxyacetone … //two times//

6 Glyceraldehyde … //two times//

7 Glycerate

8 Formazan //spelling??//

//scan in Figure 2//

Key: 1 Surface area [arbitrary units]

2 Lipoprotein concentration (g/L)

//scan in Figure 3A//

Key: 1 Free glycerol

//scan in Figure 3B//

Key: 1 Cholesterol

2 Triglycerides

3 Fraction % //two times//